Complement dependent cytotoxicity (CDC) activity of a humanized anti Lewis-Y antibody: FACS-based assay versus the 'classical' radioactive method—Qualification, comparison and application of the FACS-based approach

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ABSTRACT

The fully humanized Lewis-Y carbohydrate specific monoclonal antibody (mAb) IGN311 is currently tested in a passive immunotherapy approach in a clinical phase I trail and therefore regulatory requirements demand qualified assays for product analysis. To demonstrate the functionality of its Fc-region, the capacity of IGN311 to mediate complement dependent cytotoxicity (CDC) against human breast cancer cells was evaluated. The "classical" radioactive method using chromium-51 and a FACS-based assay were established and qualified according to ICH guidelines. Parameters evaluated were specificity, response function, bias, repeatability (intra-day precision), intermediate precision (operator–time different), and linearity (assay range).

In the course of a fully nested design, a four-parameter logistic equation was identified as appropriate calibration model for both methods. For the radioactive assay, the bias ranged from −6.1% to −3.6%. The intermediate precision for future means of duplicate measurements revealed values from 12.5% to 15.9% and the total error (β-expectation tolerance interval) of the method was found to be <40%. For the FACS-based assay, the bias ranged from −8.3% to 0.6% and the intermediate precision for future means of duplicate measurements revealed values from 4.2% to 8.0%. The total error of the method was found to be <25%.

The presented data demonstrate that the FACS-based CDC is more accurate than the radioactive assay. Also, the elimination of radioactivity and the 'real-time' counting of apoptotic cells further justifies the implementation of this method which was subsequently applied for testing the influence of storage at 4 °C and 25 °C ('stability testing') on the potency of IGN311 drug product. The obtained results demonstrate that the qualified functional assay represents a stability indicating test method.

1. Introduction

Therapeutic monoclonal antibodies (mAbs) exert their biological functions by various mechanisms which can be generally divided into two groups (for review see Adams et al. [1]). Group one consists of neutralizing mAbs that bind either to a ligand (e.g. TNFα [2]) or to a receptor (e.g. IL-6 receptor [3]) of a biological system therefore blocking this particular interaction and the subsequent cellular events. To the second group belong mAbs that first bind to target cell associated, surface expressed molecules (e.g. CD20) and then exert biological functions that can either be generated (i) via the receptor molecule (e.g. modulating downstream signaling [4]),

(ii) by mechanisms mediated by the bound mAb like complement dependent cytotoxicity (CDC) [5] and/or antibody dependent cellular cytotoxicity (ADCC) like the anti-CD20 mAb Rituximab [6], (iii) or by a combination of both (e.g. anti-Her2 mAb Herceptin [7]). Fc-mediated effector functions are initiated after the mAb has bound to its cognate antigen on the cell surface via its complementary determining regions and the Fc-region is accessible to interact with either membrane-expressed Fcγ-receptors (FcγR) [8] on effector cells or with the serum complement protein C1q. In the first case, the mAb bridges the target cell with FcγR-expressing effector cells like NK cells which then initiates ADCC [9]. In the second case designated CDC, complement protein C1q binding to the Fc-region of the mAb initiates the classical complement cascade that eventually results in lysis by insertion of the membrane attack complex in the target cell membrane (for review see Gelderman et al. [10]).

For passive anti-cancer immunotherapy, IGN311, a humanized IgG1/κ mAb specific for the tumor associated carbohydrate anti-

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gen Lewis-Y [11] has been developed. IGN311 application aims at the destruction of Lewis-Y over-expressing disseminated tumor cells by ADCC and CDC. For clinical application, drug quality is of utmost importance and must meet strict regulatory demands [12,13] requiring a series of qualified and validated assays. Besides release/stability criteria such as sterility, content and the antigen binding capability [14], the potency of the drug product has to be tested by an appropriate method [15].

For potency testing of IGN311, the CDC assay has been used because there is sufficient evidence for a correlation between the expected clinical response and activity in the functional biological assay [16]. The 'classical' radioactive CDC assay measures the release of chromium-51 from target cells into the supernatant upon prolonged incubation with various amounts of IGN311. The primary read-out is 'counts per minute' which is subsequently converted into percentage target cell lysis. Critical issues regarding the radioactive assay are the inherent problems when working with radioactivity such as dealing with regulatory and safety issues, the need of an appropriate waste disposal concept and the related costs. Therefore, as a non-radioactive alternative, a FACS-based CDC assay was developed which determines the ratio of alive versus dying cells as evaluated by the up-take of a fluorescent dye that enters only into disintegrating (dying) cells.

We here report the qualification of both assays comprising criteria such as specificity, response function, bias, repeatability (intra-day precision), intermediate precision (operator–time different), and linearity (assay range). The comparison revealed that the FACS-based assay is superior to the radioactive approach—not only in terms of accuracy of the method but also regarding economic and safety issues.

2. Material and methods

2.1. Radioactive CDC

2.1.1. Target cell preparation and radiolabeling

SKBR3 breast cancer cells were purchased from ATCC, grown in cell culture medium up to the 11th passage and frozen in liquid nitrogen ('master cell bank'). Working cell banks were generated after further three passages and freezing in liquid nitrogen. For CDC, SKBR3 cells from a working bank vial were thawed and grown in cell culture medium (CCM), RPMI 1640 containing 10%FCS (Invitrogen). The CCM was changed 18 h before the experiment. The cells were detached using a PBS solution containing 0.54 mM EDTA. The appropriate volume of the suspension (per plate: 2.5×10^6 cells) was centrifuged for 10 min at 1000 rpm. The pellet was resuspended with CCM yielding a final volume of 1 ml, transferred to a 14 ml round bottom tube and mixed with the amount of $Na_2^{51}CrO_4$ solution (Amersham, Germany) corresponding to 3.7 MBq (for one plate). Radiolabeling was performed during incubation for 2 h at 37 °C (5–8% CO_2) with rotation.

2.1.2. Sample preparation

IGN311 was pre-diluted with CCM to a concentration of 375 μg/ml. Subsequently, a 2.7-fold dilution series in CCM was prepared in a U-shaped microtiter plate to a final dilution of 1:1046. 50 μl of each dilution were transferred into the corresponding well of the test plate. For the controls, the following volumes of CCM were used: 150 μl for 'Spontaneous Lysis', 50 μl for 'Serum Zero' and 50 μl for 'Maximum Lysis'.

2.1.3. Addition of labeled cells and complement source

The suspension of radiolabeled cells was transferred into a 2 ml tube and washed three times with successive centrifugation for 30 s at 1000 rpm, removal of the supernatant and re-suspension in 1 ml CCM. After the last washing step, the cells were adjusted

to 2.5×10^6/ml. Subsequently, 100 μl of cell suspension was added to all sample, reference and control wells. Next, 100 μl of freshly thawed human normal serum (NHS) were added to all sample. NHS was taken from 3 male and 3 female healthy donors (aged 22–45 years). For the 'Maximum Lysis' control, 100 μl of freshly prepared solution containing SDS, Na_2CO_3 and Na-EDTA in dH_2O were added. Then, the plate was incubated for 1 h at 37 °C (5–8% CO_2 and $\geq 90\%$ relative humidity).

2.1.4. Harvest of supernatant and radioactivity determination

After incubation, a harvesting frame containing harvesting plugs (Skatron Molecular Devices, Germany) was placed on the test plate and the frame lever is pressed down slowly to absorb the supernatants. The harvesting plugs were transferred into the pre-labeled poly gamma test tubes (PerkinElmer, USA) and radioactivity was measured using the Gamma ounter Cobra 5005 (Canberra-Packard, Australia).

Data were evaluated with the GraphPad Prism software (version 4.1) using the four-parameter logistic (4-PL) dose response model.

2.1.5. Assay qualification and nested design

Based on a risk analysis, the following parameters were identified as critical: variability of the cell line, activity of the chromium solution, and complement source. Consequently, these parameters were nested in a 3 factor design with 2 operators, 4 days, and 2 replicates allowing the evaluation of the assay performance under ruggedness conditions. Assay parameters evaluated were specificity, response function, bias, repeatability (intra-day precision), intermediate precision (operator–time different), and linearity (assay range).

2.2. FACS CDC

2.2.1. Target cell preparation

Cell preparation was performed exactly as described for the radioactive approach. After the last washing step, the cells were adjusted to 2.5×10^6/ml.

2.2.2. Sample preparation

IGN311 was pre-diluted in cell culture medium to a concentration of 1 mg/ml. For each duplicate, one pre-dilution was prepared independently. Each pre-dilution was further diluted with CCM to a final concentration of 300 μg/ml (for the 100% sample) in a 96-well u-shaped microtiter plate. Subsequently, 2.5-fold dilution series in cell culture medium were prepared resulting in a final dilution factor of 610. 50 μl of sample and reference, respectively, were transferred into the corresponding well. As reference, IGN311 obtained from the first GMP run and stored at −80 °C was used. As controls, the following volumes of cell culture medium were added to the corresponding wells: 150 μl for 'Spontaneous Lysis' and 50 μl for 'Serum Zero'.

2.2.3. Addition of target cells and complement source

100 μl of the cell suspension were added to all sample, reference, and control wells of the test plate. Before addition, the suspension was gently homogenized with slowly pipetting using fresh tips for every row. NHS from healthy donors (aged 22–45 years) as complement source, stored at −20 °C, was brought to RT on the day of the experiment and filtered through a 0.22 μm filter. 100 μl of the filtered NHS were added to all sample, reference and serum zero wells of the test plate using fresh tipps for every row. Subsequently the plate was covered with a micro-plate lid and incubated for 1 h at 37 °C (5–8% CO_2, $\geq 90\%$ relative humidity). After incubation, the test plate was placed on ice.

2.2.4. 7-AAD staining and FACS measurement

Protected from direct light, 10 μl of a solution containing the DNA binding dye 7-AAD were pipetted to the walls micronic© tubes. Each sample was homogenized by gently pipetting and then transferred to the walls of corresponding micronic® tubes using fresh tipps for every row. Applying this technique, the 7-AAD solution was washed down with 250 μl sample and mixed. The tubes were placed on ice and incubated in the dark for 20 min. In the setup mode of the cytometer (FACSCalibur, BD), (i) the flow rate was adjusted with the serum zero sample to an average of 200–500 counts per second, and (ii) the cell population was focused in the left bottom corner in the FCS/SSC plot using the serum zero sample. Then the reference sample was measured and an FL1/FL3 plot was generated were two clearly separated populations have to be identified based on the 7-AAD signal (recorded in FL3): a low (<10 FI units) FL3 signal indicative for living cells and a high FL3 signal from dying/apoptotic cells. Prior to each measurement, samples were resuspended by vortexing. In order to guarantee constant flow conditions, after sample application, the "Acquisition" button was pressed with a delay of about 3 s. In the "Acquisition and Storage" mode, "10,000" counts "ALL", is selected.

2.2.5. Data evaluation

The cell quest pro software was used to set a gate in the FL1/FL3 plot for 7-AAD positive cells present in each sample. The region statistics with '% gated cells' (in relation to 10,000 cells counted) is displayed and used for further analysis.

2.2.6. Assay qualification

Based on a risk analysis, the following parameters were identified as critical: variability of the cell line and the complement source. Consequently, these parameters were nested in a 3 factor design as described in Section 2.1.5.

2.3. Statistical evaluation

For the radioactive CDC, the four-parameter logistic (4 PL) equation was used to describe the relationship between dilution of IGN311 and the corresponding CPM values. In order to reduce variability, duplicate dilution series were used to calculate non-linear regressions.

For the FACS-based CDC, the four-parameter logistic mathematical model was used to describe the relationship between dilution of IGN311 and the corresponding "%gated" values. Single dilution series were used to calculate non-linear regressions as preliminary experiments revealed no significant difference to duplicate dilution series.

Ratios of resulting $EC_{50\ sample}$ to $EC_{50\ reference}$ were calculated (2 replicates by 2 operators on 4 days for each sample concentration) and used to determine bias and variance components using one-way nested ANOVA.

Bias was calculated as following:

$$\%\text{R.E.} = 100 \times \left(\frac{z - \mu}{\mu} \right)$$

where z is the overall mean and μ the nominal concentration.

Repeatability (intra-day precision) for means of duplicate measurements was calculated as:

$$\%\text{C.V.} = 100 \times \left(\frac{s_w}{\sqrt{2}\mu} \right)$$

where s_w is derived from the repeatability variance component (see below).

Intermediate precision for means of duplicate measurements was calculated as:

$$\%\text{C.V.} = 100 \times \left(\frac{s_{IP}}{\mu} \right)$$

where s_{IP} is derived from the repeatability and (operator and time) variance components (see below).

The total error (accuracy) of the method was calculated as following:

$$\%\text{R.E.} = \left(\frac{100}{\mu} \right) \times [(z - \mu) \pm 2s_{IP}]$$

This provides a concentration-dependent β-expectation tolerance interval within a future observation will be located with a given probability (e.g. 95%). Variance components were calculated from ANOVA mean square errors as follows:

Repeatability variance: $\text{MS}_w = s_w^2 = (1/t)\sum_{i=1}^{t} s_i^2$ where t is the number of days and s_i^2 is the variance of the ith day.

(operator and time) variance:

$$\frac{(\text{MS}_b - \text{MS}_w)}{n} = s_b^2$$

where n is the number of replicates per day and:

$$\text{MS}_b = \frac{n}{t-1} \sum_{i=1}^{t} (z_i - z)^2$$

where z_i is the mean of the ith day.

(Operator and time)-different intermediate precision for means of duplicate measurements:

$$s_{IP} = \sqrt{\frac{s_w^2}{2} + s_b^2}$$

Data evaluation was carried out using the following software packages: Sigma Stat 3.0, Statgraphics 5.0 and GraphPad Prism 4.2.

2.4. Operational safety

While working with serum samples, protective clothing (lab coat, goggles and gloves) was used. Initial serum dilutions were prepared under laminar flow to avoid contact with aerosols. Waste was deposited according to the 'Abfallwirtschaftsgesetz'. Radioactive work and waste disposal was performed according to the national regulatory guidelines.

3. Results and discussion

3.1. Assay design

Prior to qualification, a risk assessment for each approach was performed and the critical parameters for each assay were identified. For the radioactive approach, testing included the variability of the cell line, activity of the chromium 51 solutions, and the complement source (Table 1).

As key parameter for the FACS-based assay the complement source were identified. Additionally, for practical reasons (and in contrast to the radioactive approach) the influence of various passage numbers (from 2 to 6) of the cell line (after thawing of a vial from the working cell bank) was investigated. Both parameters were tested in a nested design approach (Table 2). In a pre-qualification experiment it was confirmed that a prolonged staining time (20 min versus 4 h) with the dye 7-AAD does not influence the fluorescence read-out. The tested time period covers the maximal range that lies between the first and last sample measured during routine testing.

Table 1
Critical parameters for the radioactive CDC.

	Vial of cells thawed from working cell bank	Activity of chromium solution	Complement source
Op1d1	Vial 1	93%	NHS 1
Op1d2	Vial 2	128%	NHS 2
Op1d3	Vial 3	113%	NHS 3
Op1d4	Vial 4	111%	NHS 4
Op2d1	Vial 5	93%	NHS 1
Op2d2	Vial 6	128%	NHS 2
Op2d3	Vial 7	113%	NHS 3
Op2d4	Vial 8	111%	NHS 4

Op1d1 is operator 1 at day 1, etc.

3.2. Specificity

Upon assay setup, specificity testing for the radioactive assay was performed using Herceptin (murine IgG1/κ) as isotype matched control antibody. At the highest concentration tested, the induced cell lysis never exceed the lysis obtained with the 'NHS only' sample (data not shown).

Specificity of the FACS CDC was determined using NHS and an isotype matched control antibody (Herceptin; 375 μg/ml) as samples—none of these samples induced CDC activity (Fig. 1, Upper and Middle panel). The same experimental setup with heat-inactivated complement resulted in only background lysis; and (ii) similarly, no activity was observed when using the Lewis-Y negative HT-29 cell line as target cell line (data not shown).

Due to the handling of the target cells, apoptotic cells were generated that were identified by their increased 7-AAD signal. This population was used to define the region where dying cells caused by IGN311-mediated CDC are counted. The effect of 375 μg/ml IGN311 on the 7-AAD read-out is shown in the bottom panel—in summary, the data demonstrate that the observed accumulation of dying cells is mediated specifically by treatment with IGN311.

3.3. Response function

Three IGN311 concentrations (150%, 100% and 50%), respectively, were analysed successively. For the radioactive assay, IGN311 reference was diluted in cell culture medium resulting in a 150%, 100%, and 50% "sample", respectively, and tested against itself (100% = 375 μg ml^{-1} starting concentration). All concentrations were tested in a 2.7-fold dilution series in quadruplicates (resulting in two sigmoidal curves). For the FACS-based assay, for the evaluation of the response function in the FACS-based assay, the initial concentrations were diluted seven times 2.5-fold resulting in duplicates (resulting in one sigmoidal curve).

For the evaluation of the response function of each assay, a four-parameter logistic model was applied and the distribution of the residuals was assessed. Data from a representative experiment are shown in Fig. 2 (Panels A and B) for the radioactive method; in Fig. 3 (Panels A and B) the relationship between concentration and response are depicted for the FACS-based CDC. The 4-PL model used

Table 2
Critical parameters FACS-based CDC.

	SKBR3 cells	Complement source
Op1d1	Passage 4	NHS 1
Op1d2	Passage 4	NHS 1
Op1d3	Passage 6	NHS 2
Op1d4	Passage 6	NHS 3
Op2d1	Passage 5	NHS 3
Op2d2	Passage 5	NHS 4
Op2d3	Passage 2	NHS 4
Op2d4	Passage 2	NHS 5

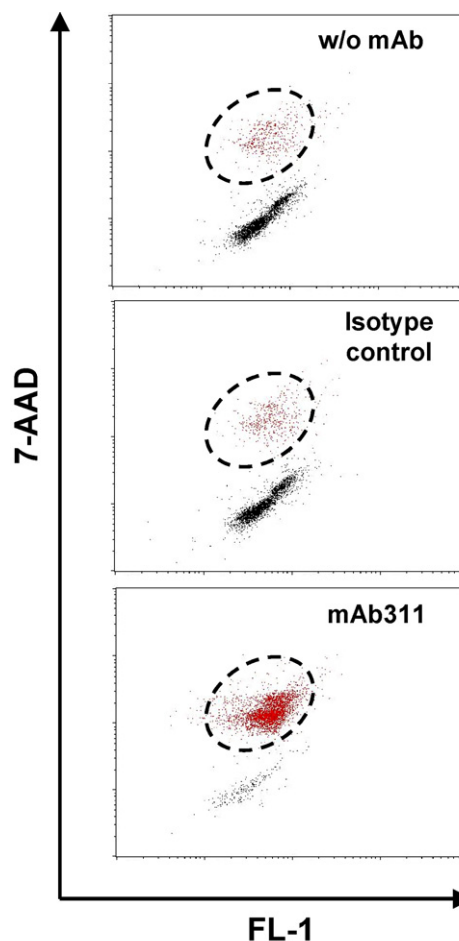Op1d1 is operator 1 at day 1, etc.

Fig. 1. FACS-CDC specificity testing. SKBR-3 cells were incubated without antibody (upper panel), with an IgG1/k isotype control mAb (middle panel), or with IGN311 (lower panel) and subsequently stained with 7-AAD. The encircled population represents the 7-AAD positive cells.

to describe the relationship between concentration and the corresponding 'cpm' and '% dead', respectively, was confirmed by the distribution of the residuals which revealed no significant trend based on visual inspection (Figs. 2 and 3, Panels B). In addition, runs test performed on all regressions revealed no deviation from linearity at $P = 0.01$.

Prior to the calculation of EC$_{50}$ values, sigmoidal curves of reference and sample had to be evaluated for parallelism or equivalence (same or similar top, bottom, and slope value for reference and sample), respectively. In the applied approach, the natural deviation of these parameters was accepted for the two sigmoidal curves compared because the curve referred to as 'sample' was similar to the 'reference' and all differences in parameters were assumed to arise by chance. These differences were calculated using the results from the 4 PL fit statistics obtained from the nested design as described above eventually providing limits for future equivalence testing evaluating parallelism between sample and reference (see compliance criteria).

3.4. EC$_{50}$ values

As final read-out of the qualification runs, EC$_{50}$ values were calculated for all sigmoidal curves (Tables 3 and 4).

3.5. Linearity

Linear regression of measured versus nominal EC$_{50}$ values revealed a linear relationship with 95% CI intervals for the slope
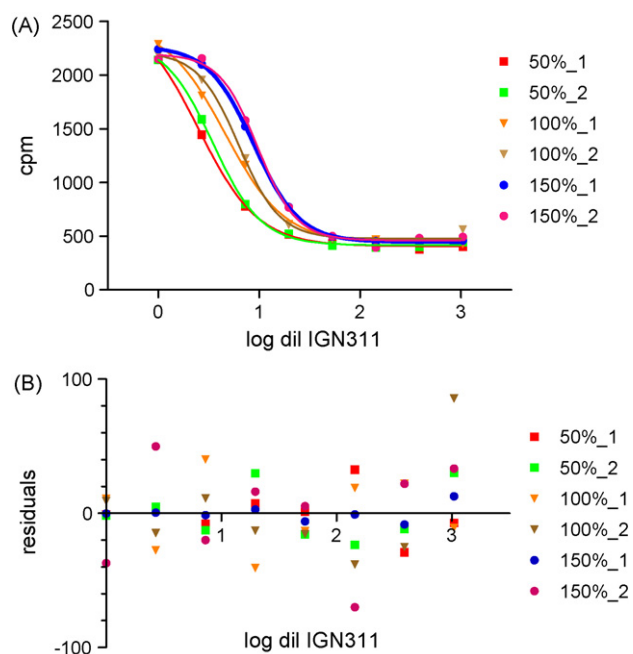
Fig. 2. Data for the 4-PL fit and the residuals are shown as mean values. Radioactive CDC. Panel A: response function. A 4PL fit model was applied to describe the relationship between concentration and 'counts per minute (cpm)'. Starting concentration of the 100% sample was 375 μg/ml. Panel B: residual distribution. The corresponding residual plot of the non-linear regression of Panel A is shown. Based on visual inspection, no significant trend in the residual distribution was observed.
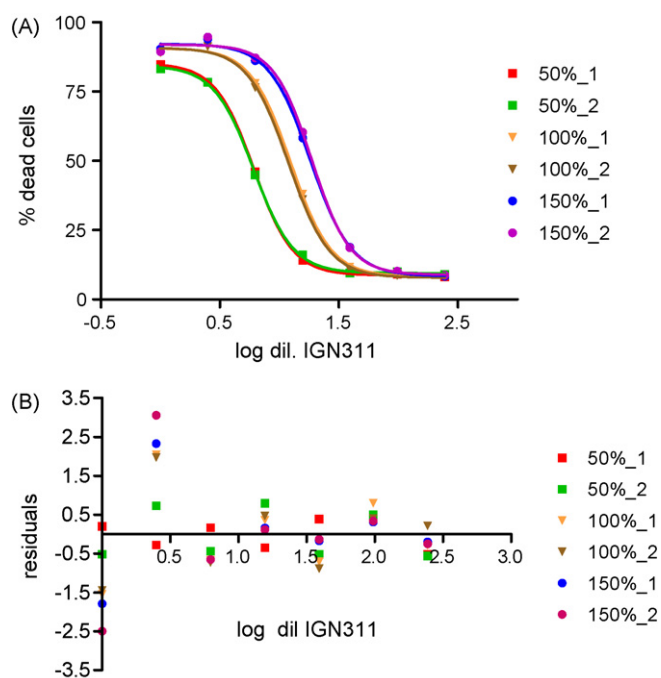

Fig. 3. FACS-based CDC. Panel A: response function. A 4-PL fit model was applied to describe the relationship between concentration and percentage dead cells. Starting concentration of the 100% sample was 300 μg/ml. Panel B: residual plot. The corresponding residual plot of the non-linear regression of Panel A is shown. Based on visual inspection, no significant trend in the residual distribution was observed.

of 0.80–1.06 and 0.99–1.11 for the radioactive-based and the FACS-based CDC, respectively. Using Durbin–Watson statistics, no indication of serial autocorrelation in the residuals at $P = 0.05$ could be detected. Furthermore, lack-of-fit test (ANOVA) qualified the linear model being adequate for the observed data in both assays; however, it should be mentioned that Bartlett's statistics indicated some degree of heteroscedasticity of residuals between the three concentrations of IGN311 at $P = 0.05$.

3.6. Bias, repeatability and intermediate precision

Linearity was confirmed by visual inspection of the residual blot, 'lack of fit' testing using one-way ANOVA, and Durbin Watson statistics (data not shown). Above EC_{50} ratios were used for the evaluation of bias, repeatability (intra-day precision), and intermediate precision (operator–time different) (Tables 5 and 6).

Table 3
EC_{50} ratios radioactive CDC.

	rep 1 (50%)	rep 2 (50%)	rep 1 (100%)	rep 2 (100%)	rep 1 (150%)	rep 2 (150%)
Op1 day1	*	*	119.3	113.9	178.9	178.6
Op2 day1	38.8	41.3	100.1	93.4	139.4	126.5
Op1 day2	46.5	62.3	84.3	107.5	145.7	150.6
Op2 day2	44.5	46.5	50.5	84.8	112.1	121.3
Op1 day3	45.4	47.2	99.4	110.2	150.7	148.1
Op2 day3	43.3	60.0	82.9	113.3	125.3	137.7
Op1 day4	53.3	64.1	76.9	112.8	142.4	141.2
Op2 day4	22.6	49.7	99.2	94.4	161.7	94.2
Grand mean		47.5		96.4		140.9

* The experiment from 'op1day1' 50% was not evaluable due to an erroneous dilution.

Table 4
EC_{50} ratios FACS-based CDC.

	rep 1 (50%)	rep 2 (50%)	rep 1 (100%)	rep 2 (100%)	rep 1 (150%)	rep 2 (150%)
Op1 day1	*	*	*	*	*	*
Op2 day1	48.4	47.7	97.3	96.8	151.2	158.1
Op1 day2	42.1	43.5	107.3	98.8	143.4	139.4
Op2 day2	50.3	46.7	86.7	96.8	165.4	161.1
Op1 day3	38.2	42.7	98.2	95.7	157.5	168.0
Op2 day3	48.7	47.5	101.5	92.6	157.2	159.9
Op1 day4	50.3	45.2	92.6	100.7	140.7	145.9
Op2 day4	45.0	45.8	91.5	88.5	134.5	130.2
Grand mean		45.9		96.1		150.9

* The experiment from 'op1day1' was excluded from the evaluation due to very low 'Top' values compared to other experiments.

Table 5
Bias, repeatability (%) and intermediate precision (%C.V.) for the radioactive CDC.

	IGN311 50%	IGN311 100%	IGN311 150%
Bias	−4.9	−3.6	−6.1
Repeatability	12.9	11.4	8.5
Intermediate precision	15.9	13.7	12.5

Repeatability and intermediate precision are shown for means of duplicate values.

Table 6
Bias, repeatability (%) and intermediate precision (%C.V.) for the FACS-based CDC.

	IGN311 50%	IGN311 100%	IGN311 150%
Bias	−8.3	−3.9	0.6
Repeatability	3.0	3.5	2.0
Intermediate precision	6.3	4.2	8.0

Repeatability and intermediate precision are shown for means of duplicate values.

Table 7
Compliance criteria.

Criterium	Radioactive CDC	FACS-based CDC
Top	907–3167 cpm	79.5–98.4% gated cells
Slope (dil.)	−3.88 to −0.46	−3.40 to −1.34
Bottom	n.a.	5.68–25.55% gated cells
Maximum Lysis	>1147 cpm	n.a.
Spontaneous Lysis	<991 cpm	<26.39% gated cells
Serum Zero	<627 cpm	<23.60% gated cells
Maximum Lysis–Spontaneous Lysis	1028–2693 cpm	n.a.
Maximum Lysis–Serum Zero	1115–2717 cpm	n.a.
Residual standard deviation	<172	<3.75
Correlation coefficient	>0.954	>0.995
Equivalence testing:		
Top difference	−615 to 1029 cpm	−7.27 to 6.98% gated cells
Slope difference	−1.14 to 2.26	−1.02 to 1.55
Bottom difference	−251 to 153 cpm	−7.65 to 8.29% gated cells

n.a.: not applicable for the particular assay.

3.7. Total error

Accuracy profiles were calculated for both assays. In Fig. 4, accuracy plots displaying the total error (accuracy = intermediate precision and bias) are shown. Bias is given as dots, error bars are 95% PI ($2s_{IP}$) for future means of duplicate measurements.

3.8. Compliance criteria

Based on the results of the qualification, for each assay a set of compliance criteria were proposed (Table 7) that will be used for routine testing. Top and slope values with the corresponding 95% CI (=2S.E.) were calculated providing confidence intervals for future testing. In a similar way, all values for mean of maximum lysis, minimum lysis, and serum zero on each plate and their differences were evaluated and limits were calculated. Additionally, the mean residual S.D. (Sy·x values) and R^2 values for all samples were computed and limits were calculated. The differences in top, bottom, and slope with corresponding CI were calculated for all "samples" versus corresponding references—their maximum and minimum confidence limits identify goal posts for three assay-based equivalence testing criteria which allow the evaluation of parallelism of future curves.

3.9. CDC activity of IGN311 stability samples

The IGN311 reference used for assay qualification comes from aliquots that were stored at −80 °C and are only thawed and used for one assay. Additionally the same aliquots were thawed and then stored for 5 months at 4 °C for stability testing and for 3 months at 25 °C ('room temperature'), respectively. The later temperature was chosen to mimic a stressed sample and to see whether this assay is capable to detect functional differences. The samples were tested with the FACS-based method resulting in a single curve after applying the 4-PL fit. All three curves met the compliance criteria defined in Table 7 and the 95% CI intervals of the EC_{50} values were extremely narrow (reference: 21.88–24.00; 4 °C sample: 19.45–22.00, 25 °C sample: 3.38–4.12). As can be seen in Fig. 5, no statistical significant difference are evident when comparing the reference to the sample stored for 5 months at 4 °C indicating that under these storage conditions the batch is stable. This finding is in accordance with the Lewis-Y ELISA where no difference regarding the binding to coated Lewis-Y-HSA between the two samples was evident (data not shown). In contrast, the stressed sample had a clearly reduced (about 6-fold) activity in the FACS-based CDC. Again, this finding correlated with reduced binding observed in ELISA (data not shown).
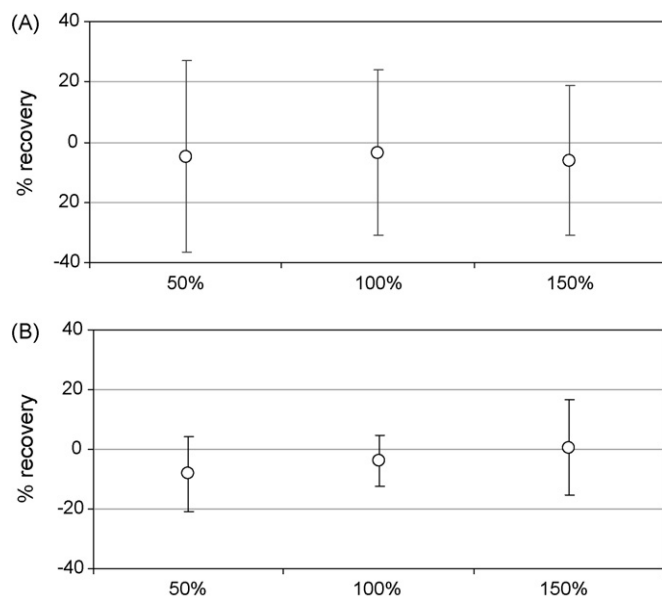

Fig. 4. Total error (accuracy = precision +/− bias). Panel A: radioactive CDC. Total error calculated for a future mean of duplicate values. Bias is shown as dots, precision (95% PI) is given by error bars. Panel B: FACS-based CDC. Total error calculated for a future mean of duplicate values. Bias is shown as dots; precision (95% PI) is given by error bars. Dashed lines represent ±20% total error.
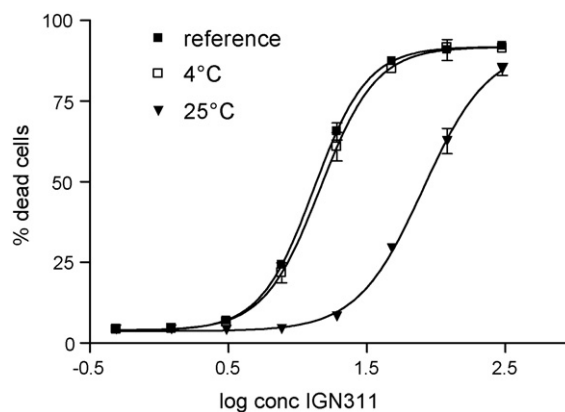

Fig. 5. FACS CDC testing of IGN311 stability samples. IGN311 reference stored at −80 °C (filled squares), IGN311 stored at 4 °C (open squares) for 5 months and IGN311 stressed at 25 °C (triangles).

4. Conclusions

The classical, well established and commercially available non-radioactive colorimetric assay suitable for quantification of cell damage/lysis is based on measurement of enzyme activity lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells by ELISA [17,18]. The advantage of the FACS-based method is that the exact number of damaged cells can be directly visualized and accurately gated/counted.

During method qualification, typical assay characteristics such as specificity, response function, accuracy, and linearity listed in FDA and ICH guidelines [12,13] were determined and used to compare the classical radioactive assay with a non-radioactive FACS-based approach. The radioactive method was found to be capable of reliably measuring the complement activating activity for IGN311 with product specifications of 50–150%. The application of duplicate measurements is recommended. Acceptance limits for critical assay performance characteristics were calculated and recommended as system suitability criteria to control the assay in routine analysis.

In contrast, the FACS-based CDC method was found to be faster, more economic and convenient. The new method is capable of more accurately measuring the complement activating properties of IGN311: earlier established IGN311 product specifications of 50–150% can therefore be narrowed significantly. Furthermore, functional alterations of the antibody can be detected earlier which may have appreciable consequences not only on product quality but also patient safety [19]. An additional benefit of the FACS-based method is the avoidance of radioactivity which is a potential hazard—also the accompanying waste disposal is a serious issue. Furthermore, due to the strict regulatory guidelines and safety issues, some working groups might not have access to a radioactive approach.

Complement activation which is often found following application of therapeutic mAbs *in vivo* is known to have a potential dual therapeutic effect On the one hand side, CDC is – beside ADCC – one of the two major effector function by which antibodies mediate their cytotoxic/cytolytic effects *in vivo*, e.g. killing of tumor cells. On the other hand, application of therapeutic mAbs may also induce unwanted side effects which were found to correlate complement activation. Recent reports have shown that infusion of the chimeric anti-CD20 mAb Rituximab can be associated with moderate to severe first-dose side effects, notably in patients with high numbers of circulating tumor cells, and was found to correlate with complement activation [20].

Monoclonal Ab IGN311 been tested regarding safety, tolerability, pharmacokinetics, and anti-tumor activity in patients with Lewis-Y positive tumors in an open-label, uncontrolled, dose escalating Phase I clinical trial [21]. In the highest dose group, three out of six patients showed drug related adverse reactions that were manageable by application of glucocorticoids, antihistamines and Serotonin (5HT3) antagonists. Whether these side effects are associated with the complement activation measured in this study will have to be evaluated.

The FACS-based method was used to evaluate the CDC activity of IGN311 stability samples. As outlined in the relevant guideline [22] there is generally no single stability-indicating method; consequently a stability indicating profile consisting of methods for identity, purity and potency is recommended. Regarding IGN311,

CDC activity belongs – together with ADCC activity [16] and inhibition of the signal transduction [4] – to the proven mode-of-action of this therapeutic antibody and therefore its accurate measurement is of high relevance. Whereas a reduction in only CDC activity indicates a problem with the Fc-part of the molecule, the correlation with reduced binding in ELISA points towards changes within the antigen binding site. Regarding these changes, the guidelines admit that the effect of glycosilation, deamidations or other microheterogeneities (e.g. methione methylation and glycation [14]) is extremely difficult to determine [18]—therefore the combination of ELISA and FACS-CDC represents an attractive tool to pin-point the nature of a potential problem.

Our data demonstrate that the qualified FACS-based CDC method is an accurate and stability-indicating assay for evaluating the quality of IGN311. Consequently this method will be implemented in our release testing program.

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